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Discrimination between plasma membrane and intracellular target sites of sphingosylphosphorylcholine

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Abstract

On the background of the emerging concept of G protein-coupled sphingolipid receptors, Ca^{2+} mobilization by sphingosylphosphorylcholine (SPPC) in intact cells and SPPC-induced Ca^{2+} release in permeabilized cells, both occurring at similar, micromolar concentrations, were characterized and compared. In intact human embryonic kidney (HEK-293) cells, SPPC rapidly increased $[Ca^{2+}]_i$ by mobilization of Ca^{2+} from thapsigargin-sensitive stores. In saponin-permeabilized HEK-293 cells, SPPC released stored Ca^{2+}, in a manner similar to but independent of inositol 1,4,5-trisphosphate. Only the action of SPPC on intact cells, but not that in permeabilized cells, was, at least in part, sensitive to pertussis toxin. In addition and most important, Ca^{2+} release by SPPC in permeabilized cells was not stereoselective, whereas in intact cells only the naturally occurring D-*erythro*-SPPC, but not L-*threo*-SPPC, increased $[Ca^{2+}]_i$. Stereoselectivity of SPPC-induced $[Ca^{2+}]_i$ increase was also demonstrated in bovine aortic endothelial cells. In conclusion, Ca^{2+} mobilization by SPPC in intact cells is independent of the previously described SPPC-gated Ca^{2+} channel on endoplasmic reticulum but probably mediated by a membrane sphingolipid receptor. Thus, SPPC can regulate Ca^{2+} homeostasis by acting apparently at two cellular targets, which exhibit clearly distinct recognition patterns.

Keywords: Sphingosylphosphorylcholine; Ca^{2+} store; Sphingolipid receptor; Ca^{2+} channel; Sphingolipid-gated

1. Introduction

The sphingolipid breakdown products, sphingosine-1-phosphate and sphingosylphosphorylcholine (SPPC), have recently been recognized as regulators of a wide variety of cellular functions (Spiegel and Milstien, 1995; Spiegel et al., 1996; Spiegel and Merrill, 1996; Meyer zu Heringdorf et al., 1997). Sphingosine-1-phosphate occurs in serum and plasma at considerable levels (Yatomi et al., 1997), and some of its cellular actions are characterized by sensitivity to pertussis toxin, EC_{50} values in the low nanomolar range and strict dependence on extracellular application, so that high-affinity G protein-coupled sphingosine-1-phosphate receptors have been postulated (Van Koppen et al., 1996a; Postma et al., 1996; Bünemann et al., 1996). Indeed, recent publications demonstrate members of an orphan G protein-coupled receptor subfamily to be activated by sphingosine-1-phosphate. For example, expression of Edg-1 in Sf9 and COS-7 cells resulted in inhibition of adenylyl cyclase and activation of mitogen-activated protein kinase, respectively, by sphingosine-1-phosphate (Zondag et al., 1998). In human embryonic kidney (HEK-293) cells, Edg-1 expression promoted specific binding of sphingosine-1-phosphate and enabled sphingosine-1-phosphate to induce cellular morphogenesis (Lee et al., 1998). Finally, H218 and Edg-3, overexpressed in Jurkat cells and *Xenopus laevis* oocytes, mediated sphingosine-1-phosphate-induced activation of serum response element-driven luciferase expression and Ca^{2+} efflux, respectively (An et al., 1997).

SPPC also appears to be an important biomolecule. This lysosphingolipid has been shown to be a potent mitogen for a variety of cell types (Desai and Spiegel, 1991). As extensively studied in Swiss 3T3 fibroblasts, where SPPC stimulates cellular proliferation to a greater extent than other known growth factors (Desai and Spiegel, 1991), SPPC has been found to stimulate arachidonic acid release (Desai et al., 1993) and DNA binding activity of activator

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protein-1 (Berger et al., 1995), to activate protein kinase C and mitogen-activated protein kinase (Seufferlein and Rozengurt, 1995a), and to induce tyrosine phosphorylation of focal adhesion kinase ($p125^{FAK}$) and paxillin as well as rearrangement of the actin cytoskeleton and focal contact assembly (Seufferlein and Rozengurt, 1995b). Furthermore, SPPC has been reported to accelerate dermal wound healing (Sun et al., 1996) and to stimulate the respiratory burst in human neutrophils (Van Koppen et al., 1996b). In a variety of cell types, SPPC induces a rapid increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Desai et al., 1993; Törnquist and Ekokoski, 1994; Okajima and Kondo, 1995; Meyer zu Heringdorf et al., 1996; Van Koppen et al., 1996a,b). With the only exception of the unique high-affinity action of SPPC ($EC_{50} \sim 1$ nM) on the muscarinic K^+ current in atrial myocytes (Bünemann et al., 1996), SPPC generally acts at low micromolar concentrations, with EC_{50} values 1–2 orders of magnitude higher than those of sphingosine-1-phosphate. Nevertheless, SPPC may also be a ligand at (a) G protein-coupled receptor(s). In this sense, An et al. (1997) reported that SPPC, similar to sphingosine-1-phosphate although with a 10-fold lower potency, induced serum response element-driven luciferase expression in Jurkat cells overexpressing H218 or Edg-3, suggesting that sphingosine-1-phosphate and SPPC share this receptor but have different affinities. However, increased mitogen-activated protein kinase activity was observed in Edg-1-expressing COS-7 cells only with sphingosine-1-phosphate but not with SPPC (Zondag et al., 1998).

On the other hand, there is ample evidence for an intracellular site of action of both sphingosine-1-phosphate and SPPC. Sphingosine-1-phosphate has been shown to directly release Ca^{2+} from intracellular stores, in a manner similar to but independent of inositol 1,4,5-trisphosphate (IP_3) and its receptors (Ghosh et al., 1990, 1994; Mattie et al., 1993). Furthermore, intracellular production of sphingosine-1-phosphate by sphingosine kinase is stimulated by platelet-derived growth factor and antigen receptors and is apparently involved in Ca^{2+} signalling by these receptors (Olivera and Spiegel, 1993; Choi et al., 1996). We have recently demonstrated that G protein-coupled receptors as well utilize sphingosine kinase to mediate intracellular Ca^{2+} signalling and that microinjected sphingosine-1-phosphate directly releases Ca^{2+} from intracellular stores (Meyer zu Heringdorf et al., 1998). All these data led to the suggestion that the sphingosine kinase/sphingosine-1-phosphate pathway may have a similar role in Ca^{2+} signalling as the phospholipase C/IP_3 pathway (Beaven, 1996). Finally, not only sphingosine-1-phosphate but also SPPC has been demonstrated to release stored Ca^{2+} from permeabilized cells (Yule et al., 1993; Kindman et al., 1994; Kim et al., 1995). The intracellular Ca^{2+}-releasing action of SPPC has been ascribed to activation of a specific sphingolipid-gated Ca^{2+}-permeable channel in the endoplasmic reticulum (Mao et al., 1996).

As both Ca^{2+}-mobilizing actions of SPPC, i.e., $[Ca^{2+}]_i$ increase in intact cells and Ca^{2+} release from intracellular stores, are observed at similar, micromolar concentrations of SPPC, it is presently unclear whether the SPPC-induced $[Ca^{2+}]_i$ increase is due to a direct intracellular Ca^{2+} channel activation by this lipid or a membrane receptor-mediated action. Notably, sphingosine-1-phosphate, but not SPPC, induced Ca^{2+} release in *Xenopus* oocytes overexpressing H218 or Edg-3 (An et al., 1997). Furthermore, although SPPC potently increases $[Ca^{2+}]_i$, it fails to activate phospholipase C in bovine aortic endothelial cells (Meyer zu Heringdorf et al., 1996) and HEK-293 cells (own unpublished observations). Therefore, aim of the present study was to characterize and compare the two probable mechanisms for Ca^{2+} mobilization by SPPC in a single cell type. We report here that SPPC rapidly releases Ca^{2+} from intracellular stores in HEK-293 cells and provide evidence that this Ca^{2+} release is not responsible for the SPPC-induced Ca^{2+} mobilization in intact cells, which most likely is mediated by a membrane receptor.

2. Materials and methods

2.1. Materials

SPPC stereoisomers were prepared from a diastereomeric mixture obtained from Sigma. Experiments using the mixture of the two isomers were performed with SPPC obtained from Matreya because of impurities in the Sigma lipid, which however were removed during the separation of the isomers. The lipids were dissolved in methanol and stored at −20°C. Before experiments, aliquots were dried in a SpeedVac concentrator and dissolved in 1 mg/ml bovine serum albumin (Meyer zu Heringdorf et al., 1996), which by itself did not increase $[Ca^{2+}]_i$ or release Ca^{2+} from permeabilized cells. $^{45}Ca^{2+}$ (51.49 μCi/mg) was purchased from DuPont New England Nuclear and IP_3 from Biomol. All other materials were from previously described sources (Meyer zu Heringdorf et al., 1996).

2.2. Separation of SPPC stereoisomers

The commercially available SPPC is a mixture of D-*erythro*-SPPC and L-*threo*-SPPC, which is formed during the semisynthetic preparation of SPPC from sphingomyelin (Van Veldhoven et al., 1989). Separation of the stereoisomers was performed by high-performance liquid chromatography (HPLC) essentially as described before (Bünemann et al., 1996), using a 250 × 2 mm column packed with 5 mm Inertsil (VDS optilab, Chromatographie Technik, Montabaur, Germany) and a Hewlett-Packard HP1050 HPLC chromatograph. The solvent gradient (solvent A: 60% chloroform, 35% methanol, 4.5% water, 0.5% ammonium hydroxide; solvent B: 30% chloroform, 60% methanol, 9.5% water, 0.5% ammonium hydroxide)

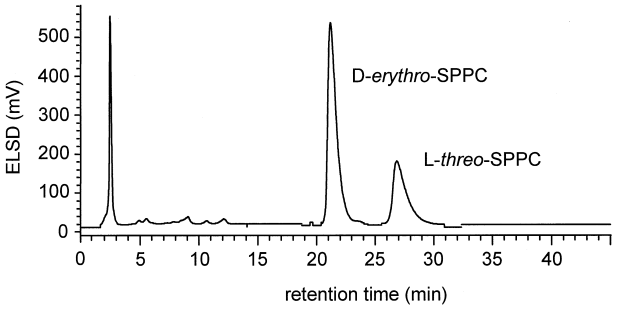

Fig. 1. Separation of SPPC stereoisomers. A diastereomeric mixture of SPPC isomers was separated by HPLC on Inertsil. Elution of the lipids was monitored with an ELSD. The peak at 2.5 min is artificial.

was run at a flow rate of 0.3 ml/min with solvent B being increased from 0% to 100% within the first 15 min. Elution of the lipids was monitored using a Sedex 55 evaporative light scattering detector (ELSD) (S.E.D.E.R.E., Alfortville, France). The elution profile shown in Fig. 1 reflects a ratio of about 70% D-*erythro*- and 30% L-*threo*-SPPC (assignment according to Bünemann et al., 1996), similar as reported before (Van Veldhoven et al., 1989; Bünemann et al., 1996), with a clear separation of the two isomers. The collected peaks were dried down, weighed and dissolved at 10 mM in methanol for storage at −20°C.

2.3. Cell culture and pertussis toxin treatment

HEK-293 cells stably expressing the human m2 muscarinic acetylcholine receptor were cultured as described in detail before (Schmidt et al., 1995). Treatment with pertussis toxin was performed with 100 ng/ml for 18 h. Primary cultures of bovine aortic endothelial cells were prepared

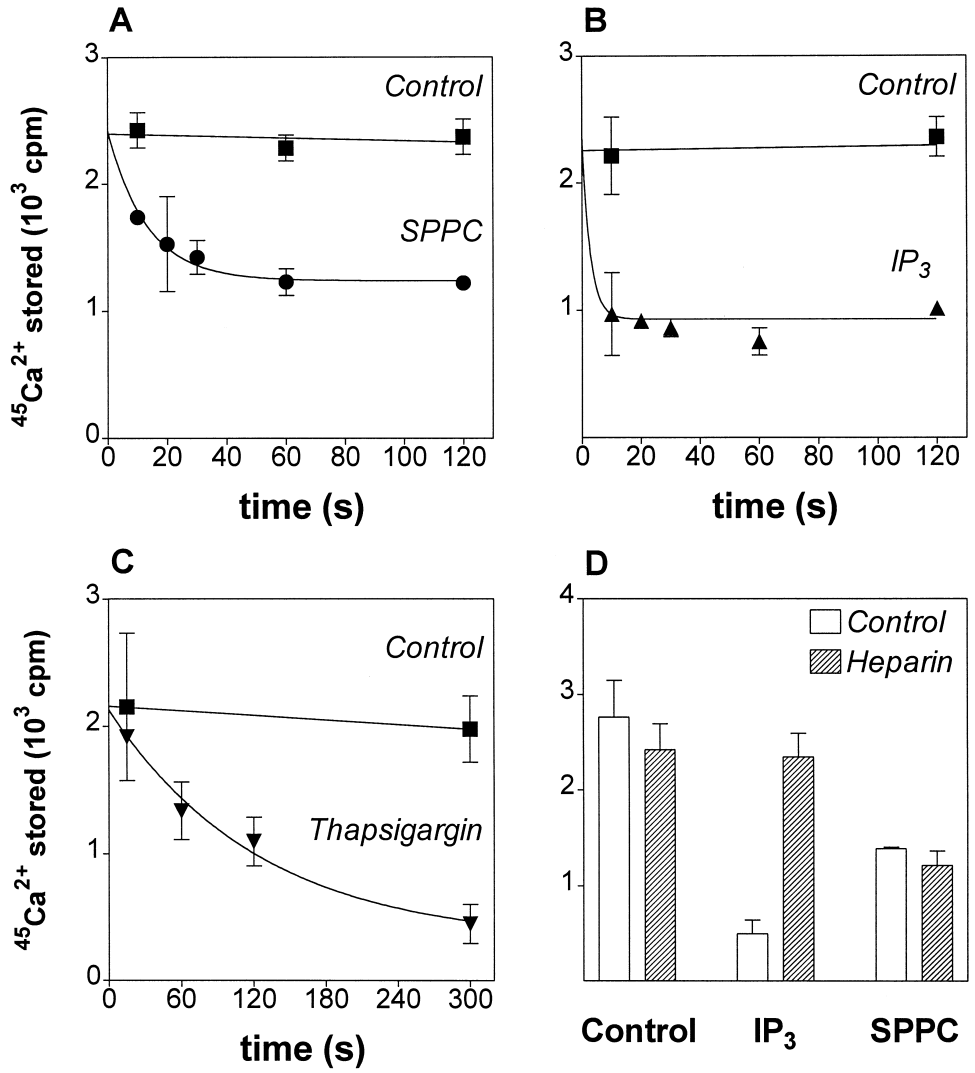

Fig. 2. Characteristics of SPPC-induced Ca^{2+} release from permeabilized HEK-293 cells. HEK-293 cells were permeabilized with saponin, loaded with $^{45}Ca^{2+}$ for 5 min in the presence of ATP and then challenged with SPPC, IP_3 or thapsigargin. (A, B and C) Time courses of Ca^{2+} release induced by 30 μM SPPC (diastereomeric mixture), 10 μM IP_3 and 1 μM thapsigargin, respectively. Note the different time scales. (D) Influence of heparin (200 μg/ml) on Ca^{2+} release induced by 5 min incubation with 10 μM IP_3 or 30 μM SPPC. Shown are representative experiments performed in triplicate (mean ± S.D.).

and maintained up to passage 10 as recently reported (Meyer zu Heringdorf et al., 1996).

2.4. Measurement of Ca^{2+} release from intracellular stores

HEK-293 cells grown on 145 cm^2 culture plates were washed twice and detached in Hank's balanced salt solution (HBSS) (118 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM D-glucose, 15 mM HEPES, pH 7.4) at 4°C. Then, the cells were washed once with ice-cold assay buffer (135 mM KCl, 2.5 mM $MgCl_2$, 0.05 mM EGTA, 20 mM HEPES, pH 7.1) and resuspended in this buffer at a protein concentration of 2 mg/ml. Permeabilization of the cells and loading with $^{45}Ca^{2+}$ was started by addition of 30 μl cell suspension to prewarmed (37°C) reaction mixture (60 μl). The final concentrations were as follows: 135 mM KCl, 2.5 mM $MgCl_2$, 0.05 mM EGTA, 0.02 mM $CaCl_2$, 20 mM HEPES, pH 7.1, 30 μg/ml saponin, 10 mM creatine phosphate, 10 U/ml creatine kinase, 1 mM ATP and 0.2 μCi/tube $^{45}Ca^{2+}$. After incubation for 5 min at 37°C, 10 μl of stimulus or control solution were added. Reactions were stopped by addition of 2.5 ml ice-cold stop solution (150 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 20 mM HEPES, pH 7.1) and rapid filtration through glass fiber filters (Whatman GF/C), followed by 3 washes with 2.5 ml each of stop solution. Radioactivity retained on the filters was determined by liquid scintillation counting. Actively stored Ca^{2+} was defined as the difference in total $^{45}Ca^{2+}$ retained on the filter after incubation in the presence and absence of ATP. Preincubation with heparin was performed by adding heparin to the reaction mixture.

2.5. Measurement of $[Ca^{2+}]_i$ in intact cells

$[Ca^{2+}]_i$ in intact cells was determined using the fluorescent calcium indicator fura-2 as described before (Schmidt et al., 1995; Meyer zu Heringdorf et al., 1996). Emission was recorded at 510 nm every 0.5 s, using a Hitachi F-2000 spectrofluorometer equipped with the appropriate software. Measurements were performed at room temperature with 10^6 cells/ml in HBSS. For measurement of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, fura-2-loaded cells were suspended in Ca^{2+}-free HBSS and EGTA (50 μM) was added immediately before start of the experiment.

2.6. Data presentation and analysis

Data are given either as mean ± S.E.M. from the indicated number (n) of independent experiments or as mean ± S.D. from replicate values of a representative experiment. Concentration–response curves were analyzed by fitting sigmoidal functions to the experimental data, using iterative non-linear regression analysis with the Prism program (GraphPad Software).

3. Results

SPPC has been shown to increase $[Ca^{2+}]_i$ upon extracellular addition in a variety of cell types (Desai et al., 1993; Törnquist and Ekokoski, 1994; Okajima and Kondo, 1995; Meyer zu Heringdorf et al., 1996; Van Koppen et al., 1996a,b). The reported EC_{50} values were in the range of 0.3–2 μM (Törnquist and Ekokoski, 1994; Meyer zu Heringdorf et al., 1996; Van Koppen et al., 1996a,b) or even higher (~5 μM; Okajima and Kondo, 1995), thus very similar to the SPPC concentrations (~3 μM) required to half-maximally induce Ca^{2+} release from intracellular stores (Ghosh et al., 1990, 1994; Kindman et al., 1994). To study the relationship between these two cellular actions of SPPC, we first analyzed HEK-293 cells, which exhibit a rapid $[Ca^{2+}]_i$ increase upon extracellular application of SPPC (Van Koppen et al., 1996a), for the occurrence and characteristics of an intracellular SPPC-regulated Ca^{2+} release and, then, asked whether these characteristics may explain the SPPC-induced $[Ca^{2+}]_i$ increase in intact cells. Specifically, we compared the two actions of SPPC for a possible stereoselectivity.

3.1. Characteristics of SPPC-induced Ca^{2+} release from permeabilized HEK-293 cells

To measure a possible SPPC-induced Ca^{2+} release from intracellular stores in HEK-293 cells, the cells were permeabilized with saponin and loaded with $^{45}Ca^{2+}$ in the presence of ATP. Under the assay conditions used, loading equilibrium was reached within 5 min (data not shown), resulting in an ATP-dependent incorporation of 240 ± 20 pmol Ca^{2+}/mg of protein ($n = 25$). Addition of IP_3 at equilibrium caused a marked release of stored Ca^{2+}, with an EC_{50} of 1.3 ± 0.21 μM (Hill coefficient 1.7 ± 0.15;

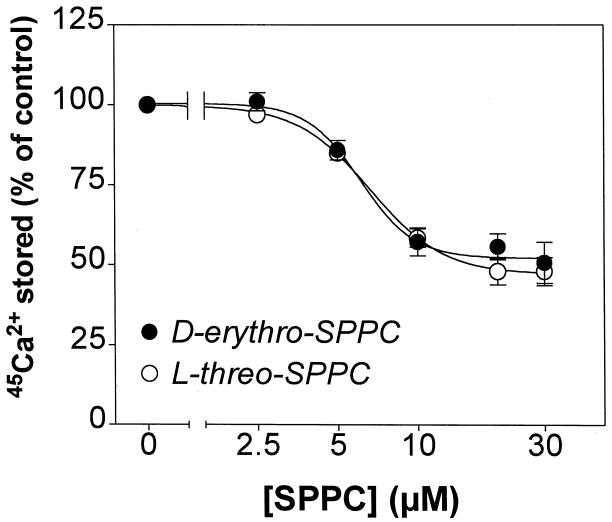

Fig. 3. Concentration–response curves of Ca^{2+} release from permeabilized HEK-293 cells induced by D-*erythro*-SPPC (●) and L-*threo*-SPPC (○). Mean ± S.E.M. from six experiments each performed in triplicate.

$n = 8$; data not shown). Addition of 30 μM of the diastereomeric mixture of SPPC to permeabilized HEK-293 cells preloaded with $^{45}Ca^{2+}$ caused a rapid release of stored Ca^{2+} (Fig. 2A). Half-maximal and maximal release (48 ± 5.5%, $n = 9$) was observed at 10 s and 60 s, respectively, with no further release being observed up to 5 min of incubation. In comparison, Ca^{2+} release induced by 10 μM IP_3, which was maximal at 10 s, was also only partial (63 ± 2.7%, $n = 17$; Fig. 2B). In contrast to IP_3 and SPPC, Ca^{2+} release caused by inhibition of the store-filling Ca^{2+} pump by thapsigargin (1 μM) proceeded very slowly and did not reach a plateau (Fig. 2C). These distinct kinetics strongly suggest that the Ca^{2+}-releasing action of SPPC in HEK-293 cells was not due to inhibition of endoplasmic reticulum Ca^{2+}-ATPase. A rapid and complete release of all stored Ca^{2+} was achieved with 1 μM ionomycin (data not shown). As reported before for other cell types (Ghosh et al., 1990, 1994; Yule et al., 1993; Kindman et al., 1994), Ca^{2+} release induced by SPPC was not inhibited by heparin, which on the other hand inhibited the IP_3-induced Ca^{2+} release (Fig. 2D), suggesting that SPPC did not act via IP_3 receptors. The SPPC-induced Ca^{2+} release fol-

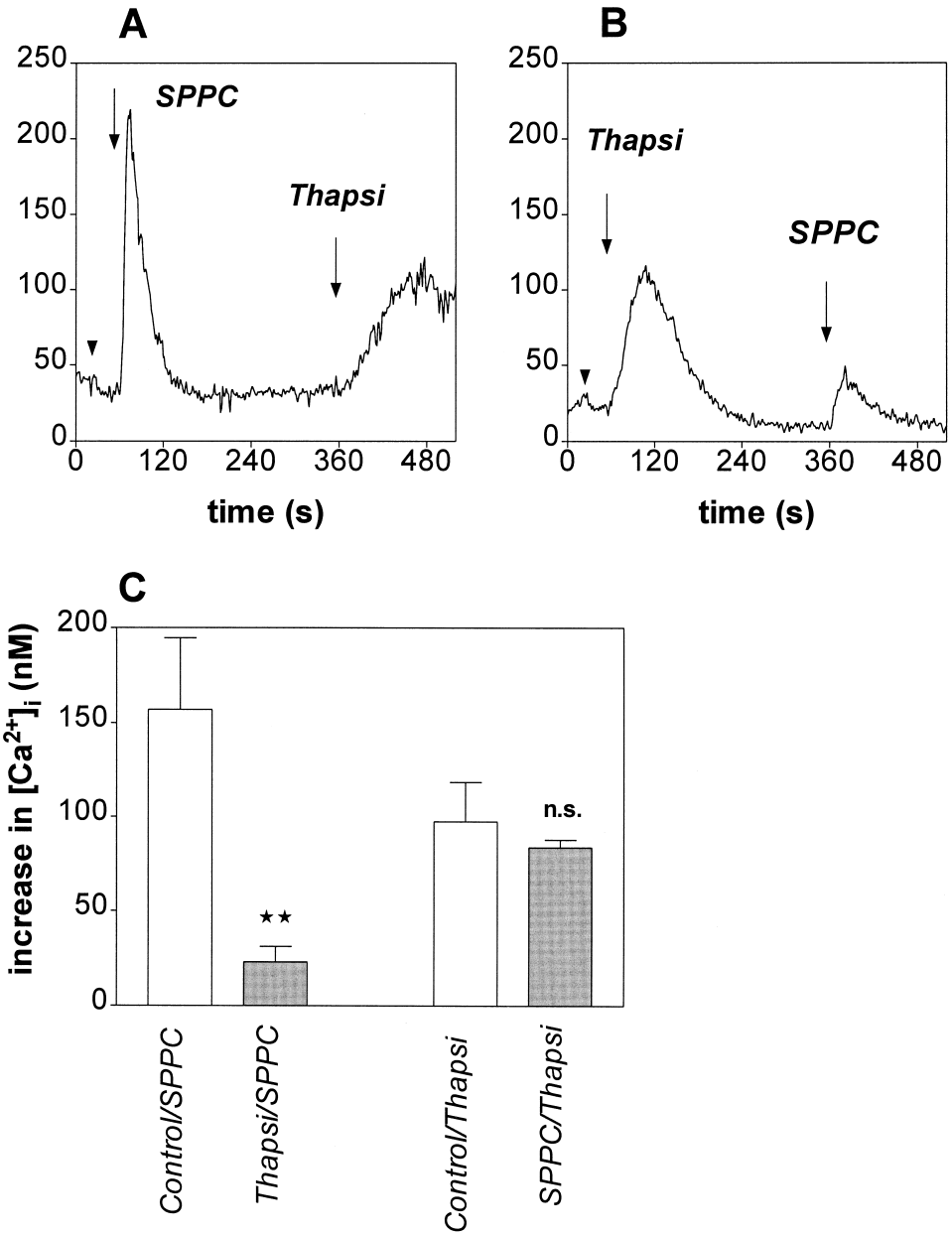

Fig. 4. Characteristics of SPPC-induced $[Ca^{2+}]_i$ increase in intact HEK-293 cells. Fura-2-loaded cells were challenged with 5 μM SPPC (diastereomeric mixture) and 1 μM thapsigargin (Thapsi) in the absence of extracellular Ca^{2+} (arrowheads indicate addition of 50 μM EGTA). A time period of 5 min was between addition of first and second stimulus. Typical traces are shown in A and B, while C displays mean ± S.D. ($n = 4$). $**$, $P < 0.01$; n.s., not significant.

lowed a sigmoidal concentration–response relationship, with an EC_{50} of 4.5 ± 0.5 μM ($n = 7$; data not shown).

Thus, in HEK-293 cells SPPC was able to induce Ca^{2+} release from intracellular stores, with similar characteristics as reported before for other cell types (Ghosh et al., 1990, 1994; Yule et al., 1993; Kindman et al., 1994; Kim et al., 1995). The question, then, was how Ca^{2+} release from intracellular stores could be related to $[Ca^{2+}]_i$ increase observed in intact cells. First, in the permeabilized HEK-293 cells stably expressing the m2 muscarinic receptor, no agonist-induced action could be observed, i.e., the muscarinic receptor agonist, carbachol, did not release any stored Ca^{2+} under the assay conditions used (data not shown). Thus, a receptor-mediated action of SPPC should

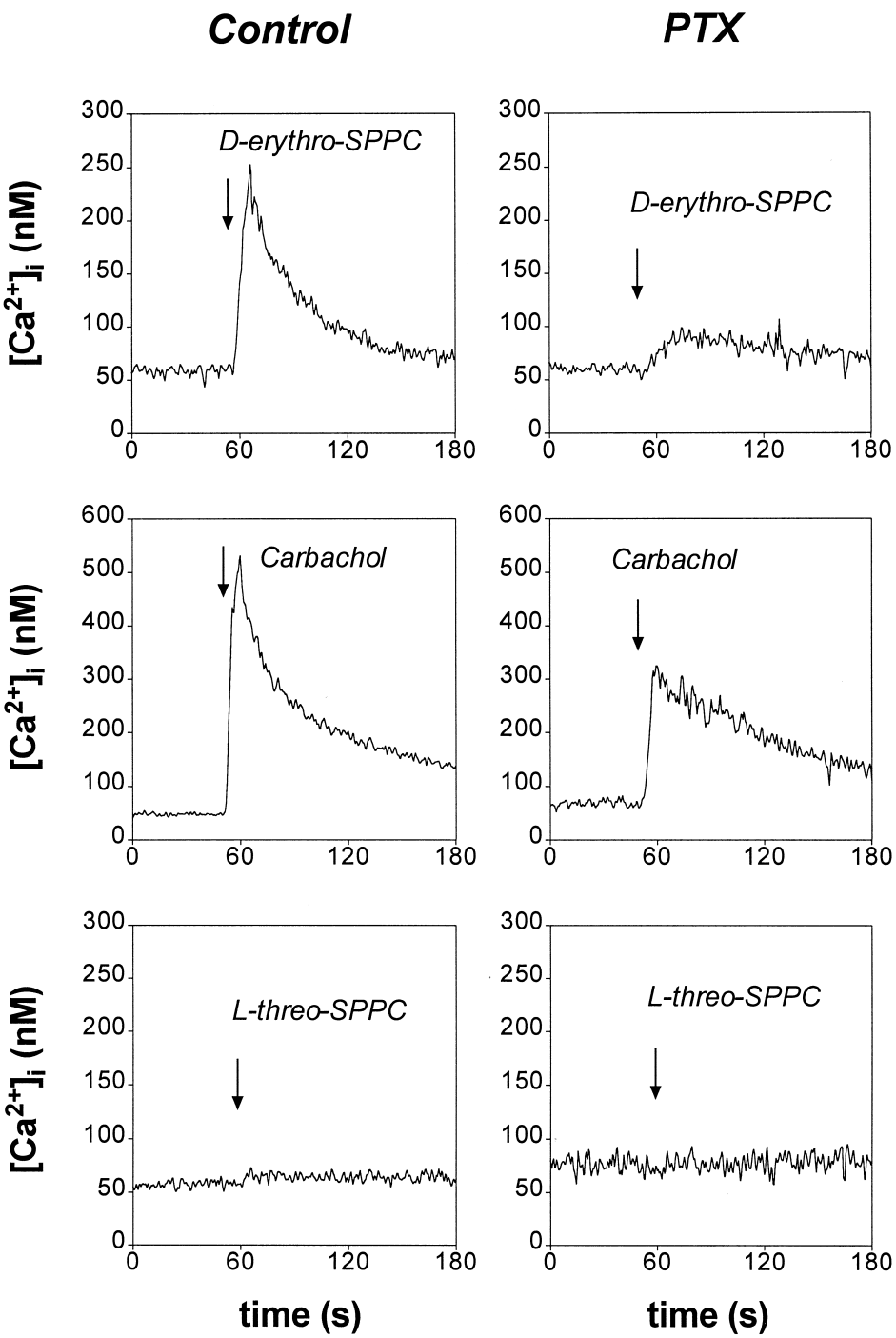

Fig. 5. Stereoselectivity and pertussis toxin sensitivity of SPPC-induced $[Ca^{2+}]_i$ increase in intact HEK-293 cells. Shown are representative recordings of $[Ca^{2+}]_i$ in fura-2-loaded HEK-293 cells expressing the m2 muscarinic receptor. Extracellular Ca^{2+} concentration was 1 mM. Control cells (left panels) and cells pretreated with pertussis toxin (PTX; 100 ng/ml, 18 h; right panels) were challenged with 5 μM D-*erythro*-SPPC, 1 mM carbachol or 5 μM L-*threo*-SPPC as indicated. Note that the time scale is different from that in Fig. 4.

probably also not be detectable in the permeabilized cell system. Second, pretreatment of the cells with pertussis toxin, which inhibited the $[Ca^{2+}]_i$ increase upon application of SPPC to intact cells (Van Koppen et al., 1996a; see below), did not affect the SPPC-induced Ca^{2+} release in permeabilized HEK-293 cells. At 30 μM, SPPC released $55 \pm 2.9\%$ ($n = 3$) of stored Ca^{2+} from pertussis toxin-treated cells, thus very similar as in non-intoxicated cells. Finally, we analyzed the SPPC-induced Ca^{2+} release for a possible stereoselectivity. As shown in Fig. 3, both the D-*erythro*- and the L-*threo*-isomer of SPPC were active, with EC_{50} values of 5.6 ± 1.5 μM and 5.9 ± 0.6 μM ($n = 4$), respectively. The amount of actively stored Ca^{2+} released by 30 μM each of D-*erythro*- and L-*threo*-SPPC was $51 \pm 7.1\%$ and $57 \pm 5.6\%$ ($n = 6$), respectively. Thus, the two stereoisomers of SPPC were equally potent and efficient in releasing Ca^{2+} from permeabilized HEK-293 cells. Interestingly, the concentration–response curves of SPPC (diastereomeric mixture or stereoisomers) were rather steep, with Hill coefficients of 4.0 ± 0.56 ($n = 15$), suggesting a cooperative action of SPPC on the putative Ca^{2+} release channel.

3.2. Characteristics of SPPC-induced $[Ca^{2+}]_i$ increase in intact cells

As reported before (Van Koppen et al., 1996a), the diastereomeric mixture of SPPC (5 μM) caused a rapid and transient increase in $[Ca^{2+}]_i$ when exogenously added to intact HEK-293 cells (Fig. 4). The rapid phase of SPPC-induced $[Ca^{2+}]_i$ increase was mainly due to release of Ca^{2+} from thapsigargin-sensitive intracellular stores. First, it could be observed in the absence of extracellular Ca^{2+}. Second, store depletion by preincubation of cells with thapsigargin (1 μM) largely diminished the SPPC-induced $[Ca^{2+}]_i$ increase. On the other hand, preincubation with SPPC did not affect the thapsigargin-induced $[Ca^{2+}]_i$ increase (Fig. 4).

The D-*erythro*-isomer of SPPC (5 μM) also caused a rapid and transient increase in $[Ca^{2+}]_i$ in intact HEK-293 cells, the time course being virtually identical to that of carbachol acting via m2 muscarinic receptors stably expressed in the HEK-293 cells used (Fig. 5). With 5 μM D-*erythro*-SPPC, peak levels of $[Ca^{2+}]_i$ were observed after 10-15 s and amounted to 171 ± 8.9 nM above basal in the presence of 1 mM extracellular Ca^{2+} ($n = 18$). As with the diastereomeric mixture, the action of D-*erythro*-SPPC could be inhibited by pertussis toxin treatment, indicating involvement of G_i type G proteins (Fig. 5). In pertussis toxin-treated HEK-293 cells, D-*erythro*-SPPC (5 μM) increased $[Ca^{2+}]_i$ by 77 ± 4.8 nM above basal ($n = 16$), i.e., to $45 \pm 2.8\%$ of that observed in control cells. In comparison, pertussis toxin treatment caused a similar, about 50% inhibition, of $[Ca^{2+}]_i$ increase induced by activation of m2 muscarinic receptors [see Fig. 5 and the work of Schmidt et al. (1995)]. Half-maximal $[Ca^{2+}]_i$ increase in intact HEK-293 cells by D-*erythro*-SPPC was observed at 1.4 ± 0.28 μM ($n = 3$) (Fig. 6).

In marked contrast to D-*erythro*-SPPC, the L-*threo*-isomer of SPPC, which was equally potent as D-*erythro*-SPPC to cause Ca^{2+} release in permeabilized cells, was virtually inactive to induce Ca^{2+} mobilization in intact HEK-293 cells (Fig. 5). At 5 μM, a concentration with which the maximal $[Ca^{2+}]_i$ increase by D-*erythro*-SPPC was observed, L-*threo*-SPPC caused only a very minor but non-significant ($P > 0.05$, $n = 6$) increase in $[Ca^{2+}]_i$ in intact cells (Fig. 6). Even at higher concentrations (> 10 μM), no further increase in $[Ca^{2+}]_i$ could be observed (data not shown). The stereoselectivity of SPPC's action on $[Ca^{2+}]_i$ in intact cells was not restricted to HEK-293 cells, but

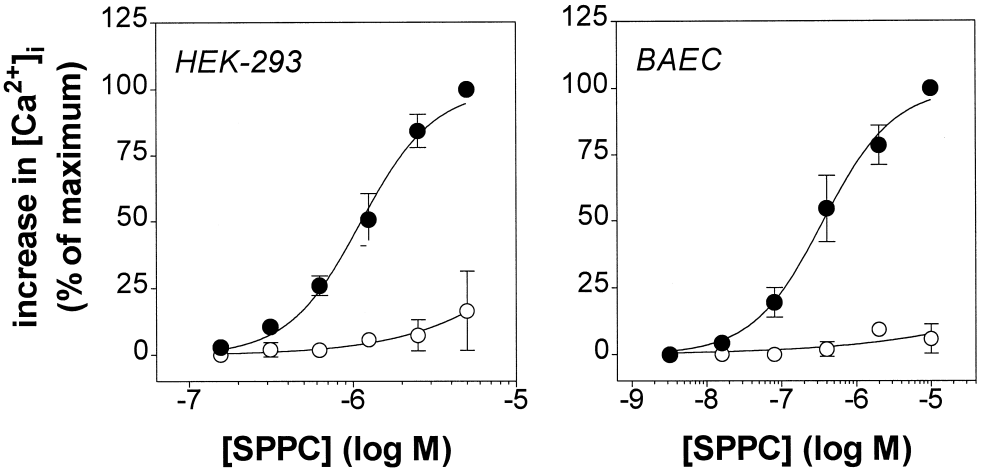

Fig. 6. Concentration–response curves of $[Ca^{2+}]_i$ increase induced by D-*erythro*-SPPC (●) and L-*threo*-SPPC (○) in intact HEK-293 cells (left panel) and bovine aortic endothelial cells (BAEC, right panel). Mean ± S.E.M. from three experiments, each performed in duplicate or triplicate.

could also be observed in primary cultures of bovine aortic endothelial cells (Fig. 6, right panel). The L-*threo*-isomer was completely inactive, while D-*erythro*-SPPC increased $[Ca^{2+}]_i$ in these cells by maximally 240 ± 25 nM ($n = 6$), with an EC_{50} of 0.40 ± 0.19 μM ($n = 3$).

4. Discussion

The lysophingolipid, SPPC, has been reported to regulate a variety of cellular functions when applied exogenously to intact cells. Specifically, SPPC has been shown to be a potent mitogen for Swiss 3T3 fibroblasts and other cell types, concomitant with a stimulation of DNA binding activity of activator protein-1, activation of protein kinase C and mitogen-activated protein kinase as well as rearrangement of the actin cytoskeleton (Desai and Spiegel, 1991; Desai et al., 1993; Berger et al., 1995b; Seufferlein and Rozengurt, 1995a,b). In addition to these rather long-term effects, SPPC has been reported to induce a rapid increase in $[Ca^{2+}]_i$ in various cell types (Desai et al., 1993; Törnquist and Ekokoski, 1994; Okajima and Kondo, 1995; Meyer zu Heringdorf et al., 1996; Van Koppen et al., 1996a,b). Some of these cellular effects of SPPC were shown to be inhibited by pertussis toxin (Törnquist and Ekokoski, 1994; Okajima and Kondo, 1995; Seufferlein and Rozengurt, 1995a; Meyer zu Heringdorf et al., 1996; Van Koppen et al., 1996a,b), and thus may be mediated by (a) G protein-coupled receptor(s), probably by a member of the recently described lipid receptor subfamily (An et al., 1997; Lee et al., 1998; Zondag et al., 1998). Another cellular target of SPPC appears to be the endoplasmic reticulum. As demonstrated for various permeabilized cell types and microsomal preparations, SPPC can cause a rapid release of actively stored Ca^{2+}, which is apparently not mediated by IP_3 receptors (Ghosh et al., 1990, 1994; Yule et al., 1993; Kindman et al., 1994; Kim et al., 1995). As both types of SPPC's action on intracellular Ca^{2+} handling, i.e., increase in $[Ca^{2+}]_i$ and release of Ca^{2+} from intracellular stores, were observed at similar, micromolar concentrations of SPPC, we searched for another criterion to discriminate these two actions of SPPC. HEK-293 cells proved to be a suitable cell type to compare receptor-mediated and intracellular actions of SPPC since in this cellular system both modes of action could be identified.

In permeabilized HEK-293 cells, SPPC induced a rapid release of stored Ca^{2+} similar as reported before for other cell types (Ghosh et al., 1990, 1994; Yule et al., 1993; Kindman et al., 1994). The maximal Ca^{2+} release induced by SPPC was in the same range of that caused by IP_3. In contrast to IP_3, however, Ca^{2+} release induced by SPPC was not inhibited by heparin. In comparison to Ca^{2+} release induced by the Ca^{2+} pump inhibitor, thapsigargin, which was similarly not inhibited by heparin (data not shown), the kinetics of Ca^{2+} release induced by SPPC were much faster. Furthermore, SPPC, like IP_3 and unlike thapsigargin, released only a limited amount of stored Ca^{2+}. Ca^{2+} release by SPPC in HEK-293 cells did not involve the ryanodine Ca^{2+} release channel since it was not inhibited by ryanodine (300 μM; data not shown), which however is apparently the case in brain microsomes (Dettbarn et al., 1995) and cardiac sarcoplasmic reticulum (Betto et al., 1997), where ryanodine receptors predominate. The EC_{50} of SPPC in inducing Ca^{2+} release from permeabilized HEK-293 cells (4–6 μM) was similar to that reported before (~ 3 μM) in a smooth muscle cell line (DDT_1MF-2 cells) and rat pancreatic acinar cells (Ghosh et al., 1990, 1994; Yule et al., 1993). From these data, we conclude that HEK-293 cells express the previously described Ca^{2+} release channel of the endoplasmic reticulum which is activated by micromolar concentrations of SPPC, apparently in a positively cooperative manner as also reported for DDT_1MF-2 and rat basophilic leukemia cells (Ghosh et al., 1990; Kindman et al., 1994).

In intact HEK-293 cells, SPPC induced Ca^{2+} mobilization from thapsigargin-sensitive intracellular stores. However, $[Ca^{2+}]_i$ increase by SPPC in intact cells was apparently independent of the above described direct action on intracellular stores. First, the active concentrations of SPPC were slightly different in the two systems. While in intact HEK-293 cells SPPC increased $[Ca^{2+}]_i$ with a maximal effect at 5 μM, 30 μM SPPC were required for maximal Ca^{2+} release. Second, pertussis toxin treatment inhibited the effect of SPPC in intact cells (by ~ 50%), without altering the Ca^{2+} release induced by SPPC in permeabilized cells. Third and most important, only D-*erythro*-SPPC was capable to mobilize Ca^{2+} in intact HEK-293 cells, whereas the L-*threo*-isomer was inactive. In complete contrast, both D-*erythro*- and L-*threo*-SPPC induced Ca^{2+} release from intracellular stores in permeabilized HEK-293 cells with identical potencies and efficacies. Thus, we demonstrate in this report for the first time stereoselectivity for one of the many cellular actions of SPPC observed upon exogenous addition of this sphingolipid at micromolar concentrations. Similar to the unique high-affinity action of SPPC on cardiac muscarinic K^+ currents (Bünemann et al., 1996), only the naturally occurring D-*erythro*-SPPC, but not L-*threo*-SPPC, mobilized Ca^{2+} in intact cells, whereas in contrast to a previous assumption (Ghosh et al., 1994) Ca^{2+} release from intracellular stores was not stereoselective.

In Swiss 3T3 fibroblasts, $[Ca^{2+}]_i$ increase induced by sphingosine was also reported to be specific for the D-*erythro*-isomer (Olivera et al., 1994). In contrast to SPPC (and sphingosine-1-phosphate), $[Ca^{2+}]_i$ increase induced by exogenously added sphingosine was only observed at high concentrations (50 μM) and with a long latency, which is assumed to be due to formation of the active metabolite, sphingosine-1-phosphate. Thus, stereoselectivity of sphingosine's action may be due to stereoselectivity

of sphingosine kinase which prefers the D-*erythro*-configuration (Buehrer and Bell, 1992) and/or to stereoselectivity of the formed sphingosine-1-phosphate.

On the background of the recent discovery of a lipid-liganded G protein-coupled receptor subfamily, the stereoselective, pertussis toxin-sensitive action of exogenously applied SPPC on $[Ca^{2+}]_i$ in intact cells is likely to be mediated by a G protein-coupled plasma membrane receptor. Nevertheless, we cannot exclude the existence of a stereoselective membrane transport mechanism for SPPC, although such a mechanism has not been described so far and cannot explain the pertussis toxin sensitivity. However, the putative G protein-coupled SPPC receptor in HEK-293 cells seems not to be one of the recently identified sphingolipid receptors, since in *Xenopus* oocytes overexpressing H218 and Edg-3 only sphingosine-1-phosphate, but not SPPC, evoked rapid Ca^{2+} efflux (An et al., 1997) and Edg-1 seems also not to be activated by SPPC (Zondag et al., 1998). Coupling of the putative SPPC receptor to intracellular signalling pathways is apparently distinct in different cell types. This concerns for example the type of G protein(s) which are involved. Although the SPPC-induced Ca^{2+} mobilization in HEK-293 and bovine aortic endothelial cells exhibited a similar strict stereoselectivity, pertussis toxin treatment completely inhibited the effect of SPPC in bovine aortic endothelial cells (Meyer zu Heringdorf et al., 1996), while in HEK-293 cells an only about 50% inhibition was observed, suggesting participation of pertussis toxin-sensitive and -insensitive G proteins in the putative SPPC receptor action. Furthermore, whereas in some cell types extracellular SPPC activates phospholipase C (Okajima and Kondo, 1995), SPPC-induced $[Ca^{2+}]_i$ increase seems to be independent of phospholipase C in others (Desai et al., 1993; Meyer zu Heringdorf et al., 1996). This has also been observed with sphingosine-1-phosphate (Chao et al., 1994; Meyer zu Heringdorf et al., 1996). In HEK-293 cells, neither SPPC nor sphingosine-1-phosphate significantly stimulated phospholipase C, measured by IP_3 mass determination or inositol phosphate accumulation in the presence of LiCl (Van Koppen et al., 1996a and own unpublished observations), and thus another, presently unknown mechanism mediates $[Ca^{2+}]_i$ mobilization by these lipid receptors. Finally, although SPPC has been identified in vivo (Seijo et al., 1994), it remains to be shown whether SPPC itself is responsible for the various extra- and intracellular actions observed at the high, micromolar concentrations or is a surrogate ligand for an as yet unidentified physiological mediator.

In conclusion, we report in a single cell type (HEK-293 cells) that SPPC both releases Ca^{2+} directly from intracellular stores and induces Ca^{2+} mobilization apparently by activating a G protein-coupled receptor. Stereoselectivity is demonstrated to be a feature of the putative plasma membrane SPPC receptor and not of the intracellular target, indicating that these two cellular sites of SPPC action exhibit distinct recognition patterns.

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